# High Velocity Chromatography of Biomacromolecules

## Technical Note

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High velocity separations are now possible for biomacromolecules due to a revolutionary innovation -- a superficially porous, silica-microsphere packing called Poroshell™. The excellent kinetic properties of these new column packings enable stable, high-resolution gradient chromatography of proteins, polypeptides, nucleic acids, DNA fragments, etc. at up to 400-times faster than conventional high performance separations. Using Poroshell technology, the chromatographer can accomplish the fastest available separation of proteins. And, Poroshell packings can be used with conventional equipment with no loss in performance.

Using a high quality liquid chromatograph like the Agilent 1100, Poroshell columns are useful for rapidly monitoring preparative separations of genetically engineered macromolecules, process control monitoring of fermentation broths or cell-reaction systems, quantitation of polymerase chain reaction fragments, monitoring protein conjugation reactions, and similar applications where very fast, reproducible separations are required.

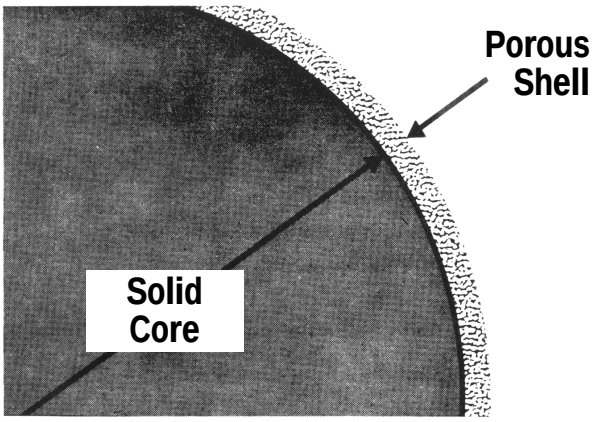
Figure 1. Structure of a typical superficially porous particle, accentuating the porous layer where macromolecules move rapidly in and out of the particle.

### Why is this a breakthrough?

Agilent has prepared superficially porous particles that represent a practical compromise of high efficiency, high speed, and low back pressure that benefit researchers doing biomolecular analysis. The basic particle is composed of a solid, ultra-pure, silica core with a thin porous silica shell. Figure 1 depicts a typical Poroshell particle. The shell consists of an ultra-pure, less-acidic "Type B" silica which has been demonstrated to be most favorable for separating polar macro-molecules, especially polypeptides and proteins. A scanning electron photomicrograph of actual Poroshell particles is shown in Figure 2. Note the consistent spherical shape of these particles.

The pore size of the outer shell is 300Å and is bonded with a sterically-protecting diisobutyl-C18 stationary phase, StableBond C18. StableBond C18 (SB-C18) is the most stable reversed-phase available. SB-C18 operates effectively at low pH (≤2) which is the region used for almost all protein separations. When used at low pH, SB-C18 is also stable to temperatures of 90°C. This is of great benefit in lowering bonded-phase viscosity so that higher flow rates may be easily achieved.

Poroshell outperforms totally porous particles because columns of totally porous particles can have constraints in separation speed because of stationary phase mass transfer limitations resulting from the relatively long diffusion times required for



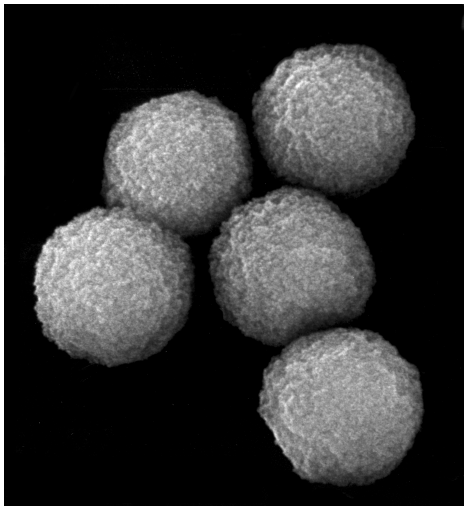


Figure2. SEM of superficially porous particles (Poroshell).

macromolecules to traverse the porous structure and interact with the stationary phase inside. Very small (<2µm) totally porous particles have been proposed as a means of reducing these mass-transfer limitations. However, optimum use of columns with <2µm particles requires special apparatus and technique because of the very narrow peaks formed. Use of these particles in conventional apparatus for isocratic separations has resulted in peaks that are broader than predicted by theory because of extra-column band-broadening effects.

## High Velocity Protein Separations

An example of the high velocity separations that achieve very high throughput is shown in Figure 3. Adequate resolution is seen at a conventional flow rate (0.5mL/min) for this 2.1 x 75mm Poroshell 300SB-C18 (Fig. 3A). Figure 3B shows the decrease in runtime achieved when operating at 3.0 mL/min. Equivalent resolution occurs in one-sixth the time. No loss of resolution and 6-times the throughput -- a dramatic example of high velocity chromatography of biomacromolecules, using Poroshell SB-C18.

Figure 4 shows a plot of plate height versus mobile-phase velocity data for insulin on a Poroshell 300SB-C18 column containing 5-µm particles with 300Å pores. The sterically protecting bonding of the Poroshell SB-C18 column provided stable operation at 60°C with a mobile phase containing 0.1% trifluoro-acetic acid (about pH 2).

Note that plate height of the insulin plot shows only modest increase with a large increase in mobile-phase velocity. This effect is evidence of the favorable mass transfer properties of the Poroshell configuration. Clearly, columns of these particles can be used at high mobile-phase velocities for very fast separation of large biomolecules without significant degradation in column resolution.

Poroshell outperforms all column material available even compared to the recently introduced ≤2µm, non-porous particles. While columns of non-porous particles theoretically offer excellent mass transfer characteristics, they suffer from problems similar to those previously described for very small, totally porous particles. Special apparatus and operating techniques are required to attain benefit from non-porous particles, because standard instrumentation broadens the very narrow peaks formed by these materials. Further, small, non-porous particles have very low surface area available for sample interaction and are limited in their sample loading capability.

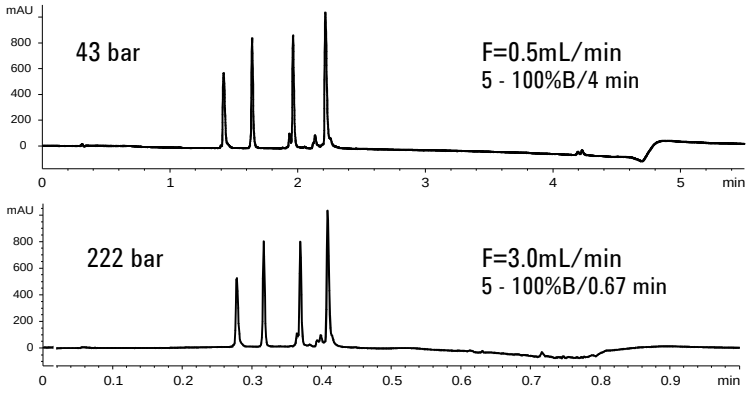



Figure 3. Comparison of flow rate in ultra-fast analysis of proteins using Poroshell. Column: 2.1 x 75mm, 5µm, Poroshell 300SB-C18; mobile phase: A=acetonitrile, 0.1%TFA (5:95); B=acetonitrile, 0.07% TFA (95:5); temperature: 35°C.

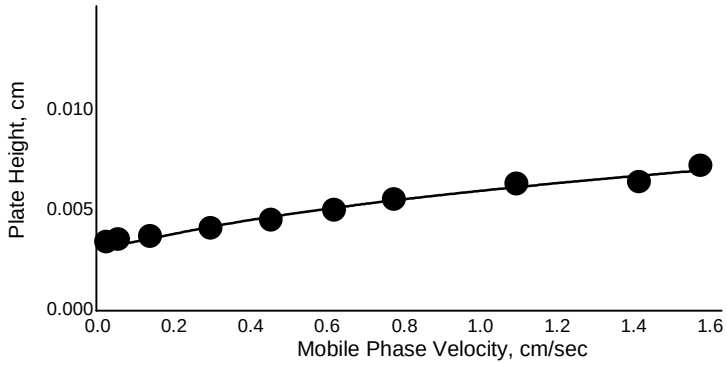



Figure 4. Plate height versus mobile-phase velocity plots for insulin. Column: Poroshell 300SB-C18, 2.1 x 75mm, 5µm; mobile phase: acetonitrile, 0.1%TFA (28:72); Temperature: 60°C; Detection UV (214nm) sample: in 6M guanidine HCl.

## Applications

Fig. 5 is an illustration of the speed and efficiency of a Poroshell column in separating protein mixtures. This synthetic mixture of nine proteins was separated by gradient chromatography, in less than 2 min., with excellent peak shape, and with resolution exceeding 1.5 for all peaks.

Even faster separations of such compounds are possible at higher mobile phase velocities (higher flow rates) and elevated column temperatures, as shown in Figure 6. High temperature improves the kinetics of the separation and lowers the operational back pressure. This separation of proteins was completed in about 20 seconds, again, with good peak shapes. At this high mobile-phase flow rate (4.0 ml/min for a 2.1 mm I.D. column; velocity ~3.2 cm/s), the pressure of the column was 295 bar, well within the operating range of modern pumping systems. For comparison, the same linear velocity on a column 4.6mm in diameter would be nearly 20 ml/min!

Another dramatic example of pushing the limits of high velocity chromatography is shown in Figure 7. Here, four proteins are separated at a conventional flow rate of 0.5ml/min on a 2.1 x 75mm column. (The same velocity on a 4.6mm i.d. column would be 2.4ml/min.) As the linear velocity (flow rate) of the mobile phase is increased, the gradient time was adjusted to keep the same rate of change for each flow rate. At 4 ml/min (corresponding to 19.2ml/min), the resolution is essentially the same and the throughput is 8-times faster. The final separation is completed in approximately 20 seconds.

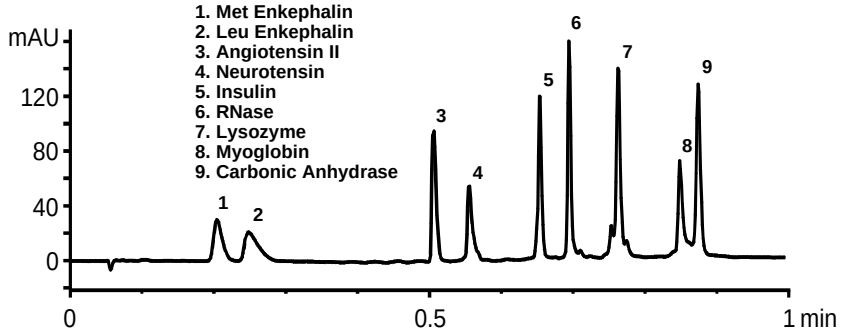



Figure 5. Rapid separation of protein mixture. Column: 2.1 x 75mm, 5µm, Poroshell 300SB-C18; mobile phase: A=acetonitrile, 0.1%TFA (5:95); B=acetonitrile, aqueous 0.085% TFA (95:5); gradient: 2 - 65% B in 2.0 min; flow rate: 2.0mL/min; temperature: 35°C. Reproduced with permission (see reference).

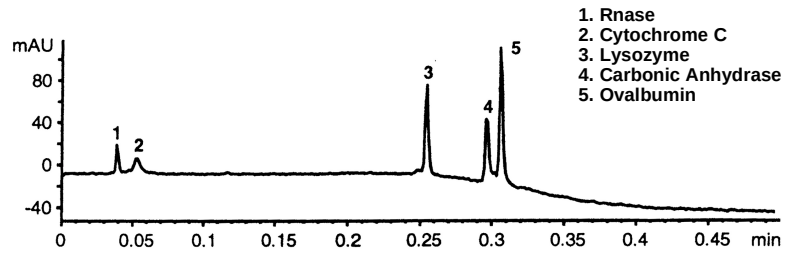



Figure 6. Rapid separation of protein mixture. Column: 2.1 x 75mm, 5µm, Poroshell 300SB-C18; mobile phase: A=acetonitrile, 0.1%TFA (5:95); B=acetonitrile, aqueous 0.085% TFA (95:5); gradient: 2 - 65% B in 2.0 min; flow rate: 2.0mL/min; temperature: 35°C. Reproduced with permission (see reference).

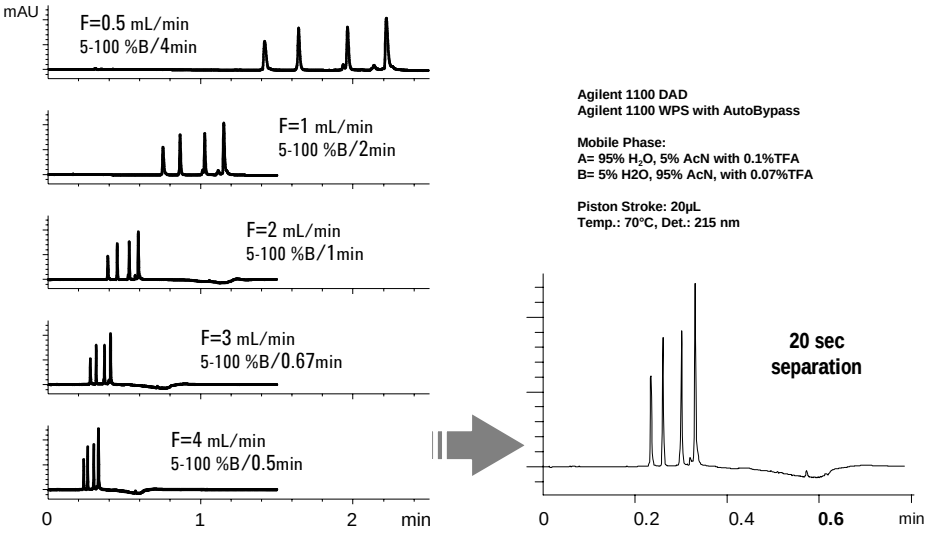



Figure 7. Protein separation in seconds; effect of flow rate on ultra-fast protein analysis. Column: 2.1 x 75mm, 5µm, Poroshell 300SB-C18; mobile phase: A=acetonitrile, 0.1%TFA (5:95); B=acetonitrile, 0.07% TFA (95:5); gradient: 5 - 100% B; flow rate: 2.0mL/min; temperature: 70°; detect.: UV (206nm).

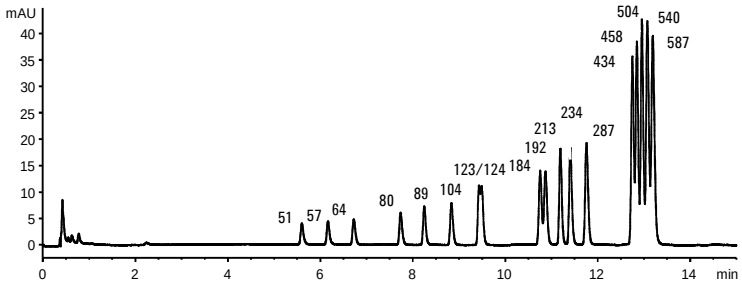



Figure 8. Separation of pBR322/HaeIII digest using a Poroshell column. Column: 2.1 x 75mm, 5µm, Poroshell 300SB-C18; mobile phase: A=100mM, pH 7, triethylamine acetate, 0.1M EDTA, B=A+acetonitrile (50:50); gradient: 17.5%B to 37.5%B in 15 min; flow rate: 0.4mL/min; temperature: 50°C; detection: UV (254nm). Reproduced with permission (see reference).

Rapid separations on more complicated mixtures also can be performed with Poroshell columns, as shown in Fig. 8. This separation of a pBR322 Hae III digest with 51 to 587 base pairs was separated sufficiently in less than 14 min. to identify all of the components in this mixture.

Poroshell columns also can be effectively used for rapid separations of polymer oligomers, and other macromolecules of this type. Figure 9 shows a gradient chromatogram of Triton X-114 that was completed in less than 3 min., with individual components clearly resolved and visible for identification.

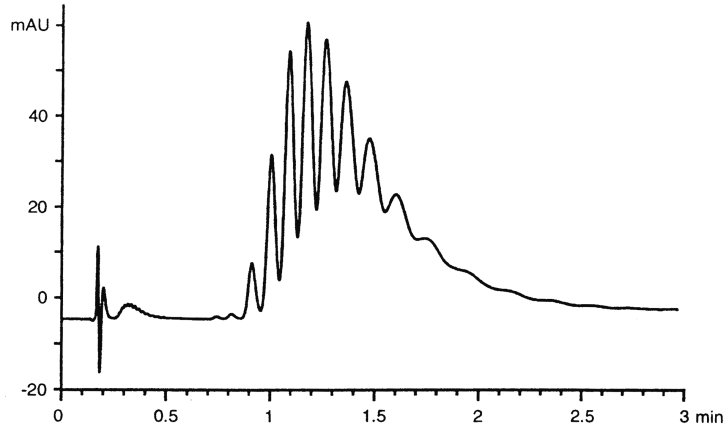



Figure 9. Separation of Triton X-114 oligomers using a Poroshell column; Column: 2.1 x 75mm, 5µm, Poroshell 300SB-C18; mobile phase: A=methanol-water (45:55), B=methanol; gradient: delay, then 0-5%B in 2 min; flow rate: 0.5mL/min; temperature: 24°C; Detection: UV (225nm). Reproduced with permission (see reference).

## Summary

The introduction of 5µm Poroshell particles marks the dawn of a new age of high velocity chromatography using conventional HPLC instrumentation. Now the chromatographer can enjoy the benefits of extremely rapid biomacromolecular analysis, without loss of efficiency. Larger peptides, proteins, and DNA can all be separated with very short runtimes, as a result of this state-of-the-art technology. Any time separation is limited by diffusion of large molecules, and rapid analysis is critical, use Poroshell technology.

## Reference

J.J. Kirkland, F.A. Truzkowski, C.H. Dilks Jr., and G.S. Engel. *J. Chromatogr.* **890** (2000) 3-13.

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